UNITED STATES PATENT AND TRADEMARK OFFICE

I, Charles Edward SITCH BA,

Managing Director of RWS Group Ltd UK Translation Division, of Europa House, Marsham Way, Gerrards Cross, Buckinghamshire, England declare;

1. That I am a citizen of the United Kingdom of Great Britain and Northern Ireland.

2. That the translator responsible for the attached translation is well acquainted with the German and English languages.

3. That the attached is, to the best of RWS Group Ltd knowledge and belief, a true translation into the English language of the accompanying copy of the specification filed with the application for a patent in Germany on June 20, 2003 under the number 103 28 080.4 and the official certificate attached thereto.

4. That I believe that all statements made herein of my own knowledge are true and that all statements made on information and belief are true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application in the United States of America or any patent issuing thereon.

For and on behalf of RWS Group Ltd

The 26th day of April 2010

FEDERAL REPUBLIC OF GERMANY

Priority Certificate
for the filing of a Patent Application

File Reference: 103 28 080.4

Filing date: 20 June 2003

Applicant/Proprietor: Dade Behring Marburg GmbH,
35001 Marburg/DE

Title: Novel surface protein (HBsAg) variant of the hepatitis B virus

IPC: C 07 K, C 12 Q, C 12 N

The attached documents are a correct and accurate reproduction of the original submission for this application.

Munich, 27 July 2005
German Patent and Trademark Office
The President
pp

[seal of the German Patent and Trademark Office]

[signature]
Remus

Novel surface protein (HBsAg) variant of the hepatitis B virus

The invention relates to sequences of a novel mutant or variant of the hepatitis B surface antigen (HBsAg) and to methods for detecting this genomic and protein variant as well as antibodies in patient samples which are directed against it.

The novel sequences lead to 5 amino acid substitutions, which have not yet been disclosed in the prior art, in the hepatitis B surface antigen, HBsAg, i.e. in amino acid positions 115 to 181 of the amino acid sequence of the surface antigen, with 4 substitutions being located in the region of the a determinant (aa 101 to aa 180) and 1 substitution in the direct vicinity thereof (aa 181).

The invention also relates to immunochemical detection methods for simultaneously detecting this novel HBV variant together with known variants/subtypes, as well as to the use of the novel sequences in combination with known sequences for simultaneously detecting HBV-specific antibodies. The antigen or antibody determination can in each case be carried out in a test assay which differentiates or does not differentiate.

Finally, the invention also relates to the detection of the corresponding nucleic acids with the aid of nucleic acid tests (e.g. polymerase chain reaction, PCR) using suitable primers, as well as to the use of the novel amino acid sequences for producing vaccines.

As is known, the hepatitis B virus is the agent responsible for a large number of disease courses, ranging from mild inapparent infections through to liver inflammations which are caused by viral infections (viral hepatites), which are chronically active and which take a fulminating course.

With an estimated 400 million persons being affected, chronic infection with HBV constitutes a global health problem (Lee, N. Engl. J. Med. 337; 1733-1745 (1997)).

Active immunization (stimulating the antibody response by administering antigen) and passive immunization (produced by injecting preformed antibodies) are regarded as being the most suitable prophylaxis for the HBV infection which can frequently be encountered world-wide.

HBV belongs to the Hepadna viruses and constitutes a virus particle having a diameter of 42 nm which consists of a core and an envelope. The genome of the virus is a double-stranded, circular DNA sequence of about 3200 nucleotides which encode at least six different viral genes (Tiollais et al., Nature 317: 489-495 (1985)).

Four open reading frames are available for forming the viral protein.
The S gene contains the information for the HBV surface antigen (HBsAg), which is also termed small protein (S). In addition, there are also larger forms which are designated large protein (L) and middle protein (M). All three proteins possess in common the S-HBsAg sequence comprising 226 amino acids (Gerlich et al., Viral Hepatitis and Liver Disease, Hollinger et al., William-Wilkens, Baltimore, MD, pages 121-134 (1991)). The protein regions upstream of the small HBs are also termed pre-S1 and pre-S2, comprise 108 and 55 amino acids, respectively, and are both present in the L protein (389 amino acids), while the M protein only comprises pre-S2 together with S antigen (281 amino acids). The pre-S proteins exhibit different degrees of glycosylation and carry the receptors for recognizing the liver cells.

The C gene carries the information for the nucleocapsid protein hepatitis B core antigen (HBcAg). The translation of this protein can already start in the pre-C region and leads to the formation of hepatitis B e antigen (HBeAg). The folding and immunogenicity of HBeAg differs from that of HBcAg. In contrast to HBcAg, HBeAg occurs in free form in serum and, in connection with positive detection, is regarded as an indicator of the formation of HBcAg and consequently of the formation of infectious viral particles.

The reverse transcription DNA polymerase which is present in the virus particle is encoded by the P gene, and the possibility is debated of the transactivator X gene having a causative role in the development of HBV-associated primary liver cell carcinomas.

The viral replication cycle of HBV includes an intracellular pregenomic RNA which is reverse transcribed, in the viral nucleocapsid, into that in DNA. Since the reverse transcriptase DNA polymerase which is intrinsic to the HBV does not possess any proof-reading capability, incorrect nucleotides are incorporated at a relatively high frequency. As a consequence, HBV exhibits a mutation rate which, at approx. 1 nucleotide/10 000 bases/infection year, corresponds to about 10 times the rate exhibited by other DNA viruses (Blum, Digestion 56: 85-95 (1995); Okamoto et al., Jpn. J. Exp. Med. 57: 231-236 (1987)). In addition, deletions and insertions also occur quite frequently (Carman et al., Lancet 341: 349-353 (1993)).

The resulting variability of HBV is manifested, inter alia, in the occurrence of 9 serologically defined subtypes (Courouce et al., Bibliotheca Haematologica 42: 1 (1976) and a total of at least 6 different genotypes, which are designated A to F (Fig. 1) and are dispersed geographically. (Norder et al., J. Gen.

Virol. 73: 3141-3145 (1992), Norder et al., Virology 198: 489-503 (1994)). In addition, a number of mutants in which 1 amino acid or more has/have been substituted, or is/are missing or supernumerary, have been described.

Aside from mutations which take place naturally (Cooreman et al., Hepatology 30: 1287-1292 (1999)), administering HBV immunoglobulins and/or an antiviral therapy (e.g. using lamivudine) can exert a selection pressure which leads to an increase in the occurrence of what are termed escape mutants and can markedly increase the probability of the appearance of HBV mutants (Terrault et al., Hepatology 28: 555-561 (1998); Tillmann et al., Hepatology 30: 244-256 (1999); Hunt et al., Hepatology 31: 1037-1044 (2000).

Not all HBV mutations result in replication-capable viruses and there is frequently coexistence with replication-capable virus, a situation which also limits the precision of the sequencing of isolated DNA or even leads to the failure of PCR, cloning procedures and subsequent sequencing to recognize altered sequences when these latter make up quantitatively less than 10% of the total DNA (Cooreman et al., J. Biomed. Sci. 8: 237-247 (2001).

It is consequently advantageous to isolate mutants, with the subsequent identification and characterization of individual mutants possibly leading to improved vaccines and diagnostic agents.

After an infection with HBV, the immune response is principally directed against what is termed the a determinant, as a region of the S protein which is common to all hepatitis B viruses, which region is located on the surface of the virus particles (Gerlich et al., see above) and constitutes the most

heterogeneous part of the B cell epitopes of the S gene.

According to the present state of knowledge, a total of at least 5 partially overlapping epitopes on the a determinant between amino acid positions 101 and 180 are assumed to be binding sites for antibodies (Figs. 1 and 2), as has been demonstrated by using monoclonal antibodies (Peterson et al., J. Immunol. 132: 920-927 (1984)).

These epitopes are chiefly complex conformational epitopes which are stabilized by several disulfide bridges. Some sequence epitopes, which can be produced using synthetically prepared cyclic peptide structures, are also present.

99% of so-called "protective antibodies", which circulate in serum after a natural infection with HBV, are directed against the very immunogenic a determinant of the HBV (Jilg, Vaccine 16: 65-68 (1998).
The widespread use of immunization with vaccines which have either been isolated from human serum or prepared recombinantly, and the administration of hepatitis B immunoglobulins which contain human HBV-specific antibodies, are based on this fact. Both prophylactic strategies are based on the neutralizing effect which HBs-specific antibodies display after binding to the "a loop epitope" (Carman et al., Hepatology 24: 489-493 (1996), Muller et al., J. Hepatol. 13: 90-96 (1991) and Samuel et al., N. Engl. J. Med. 329: 1842-1847 (1993)).

In a similar manner, diagnostic agents which are widely used nowadays are based on the binding of a determinant-specific antibodies with epitopes of the a determinant.

Thus, in the case of the HBsAg determination, using

immunochemical determination methods, which is employed world-wide in the field of blood donation, HBV surface antigen which is circulating in the serum of donors is detected using antibodies (of polyclonal or monoclonal origin) which are directed against the a determinant and, if the result is positive, the relevant donated blood is discarded in order to prevent iatrogenic HBV infections due to HBV-contaminated blood. Another application of the HBsAg determination lies in detecting an existing acute HBV infection. Conversely, a positive result when determining HBs-specific antibodies (anti-HBs) in the blood of test subjects demonstrates that either a natural infection has taken its course or that a vaccination which has been carried out has been successful.

Finally, nucleic acid testing, e.g. by means of the polymerase chain reaction (PCR), is also based on using primers (starters) which are specific for the HBV nucleotides.

Due to the central role which the a determinant in active immunization (vaccination with HBV antigen), passive immunization (protection by means of HBV-specific immunoglobulins), detection of the success of a vaccination or of an HBV infection which has taken place (both by means of determining HBsAg-specific antibodies, i.e. anti-HBs) and, finally, safety in the field of blood donation (HBsAg determination and PCR), it is understandable that the appearance of mutants, and also new variants, is followed with great attention in specialist circles.

As a consequence, novel mutants and/or variants which were altered in the a determinant of the HBV, but which were capable of replication, could be of interest both in connection with prophylaxis and in connection with diagnosis (Brind et al., J. Hepatol. 26: 228-235

(1997), Fischer et al., Transplant Proc. 31: 492-493 (1999), Ghany et al., Hepatology 27: 213-222 (1998), Protzer-Knolle et al., Hepatology 27: 254-263 (1998), Carman et al., Gastroenterology 102: 711-719 (1992) and Coleman et al., WO 02/079217 A1, (2002)).

While there is no sharp differentiation of variants and mutants of HBV, a proposal in this regard is applied widely (Carman, J. Viral Hepat. 4 (suppl. 1): 11-20 (1997).
According to this proposal, the designation "variant" should be used for naturally occurring subtypes which appear without any known interference due to selection pressure (antiviral therapy and/or immunoglobulin administration) and exhibit a geographic dispersion pattern.

The characterization and subsequent classification of the subtypes is effected using monoclonal antibodies and is based on a change in the reaction patterns due to one or a few amino acid(s) being substituted. Amino acid positions 122 and 160 of the most widespread HBV sequence: aa 122 and aa 160 = lysine, K, constitute the basis for the classification.

All the serotypes contain the group-specific a determinant while the aa 122 and, in addition, 133 and 134 determine the d or r subtype and aa 160 determines membership of the w or r subtype. On this basis, HBV subtypes can be roughly divided into adr, adw, ayr and ayw, which subtypes can be further differentiated into at least 9 sub-subtypes: ayw1, ayw2, ayw3, ayw4, ayr, adwr2, adw4, adrq+ and adrq- (Swenson et al., J. Virol. Meth. 33: 27-28 (1991), Blitz et al. J. Clin. Microbiol. 36: 648-651, Ashton-Rickardt et al., J. Med. Virol. 29: 204-214 (1989)).

Since this classification is based on serologic

reactivity, every typing does not necessarily have to denote variability at the amino acid level, for which reason preference is given to genotyping at the S gene level (Ohba et al., Virus Res. 39: 25-34 (1995).

For reasons not yet known, subtypes appear in particular geographic and ethnic patterns.

According to Carman, the designation mutation should be reserved for variants which arise exclusively under selection pressure such as vaccination or antiviral therapy. Many mutations have already been described, with a number of them giving rise to diagnostically incorrect findings (Carman et al., Lancet 345: 1406-1407); the aa substitutions which are mentioned below are cited as examples of these mutations:

Consensus:	aa Position	Mutant:
I	110	V
P	111	T
T	114	S
T	116	S
P	120	T/S
T	123	A/N
I/T	126	A/S
Q	129	H/R
K/M	133	L
T	143	M/L
D	144	H/A/E
G	145	R/A
A	157	R

and also cysteine substitutions in aa positions 107, 124, 137, 147 & 149.
(Coleman, see above; Okamoto et al., Pediatr. Res. 32: 264-268 (1992); Zhang et al., Scand. J. Infect. Dis. 28: 9-15 (1996); Zuckermann et al., Lancet 343: 737-738 (1994)).

Surprisingly, an atypical reaction pattern of hepatitis markers was found in a sample taken from a patient from France (internal number: 119617) who had contracted inflammation of the liver.

Aside from the clinical picture involving an increase in the liver values which were typical for such an infection, IgM class hepatitis core antibodies which were detected also indicated an acute HBV infection, without, however, HBsAg being detected when using an approved high-performance HBsAg ELISA.

A PCR which was carried out surprisingly gave a positive result with the sample, and sequencing led, entirely unexpectedly, to the nucleotide sequence depicted in Figs. 3 and 4 and to the amino acid sequence depicted in Figs. 5 and 6, which both unexpectedly led to the substitution pattern described.

It is clear from these sequences that it is, entirely unexpectedly, not a matter of a point mutation, i.e. the substitution of a few nucleotides, and not a matter, either, of a subtype which might possibly be characterized serologically, since a total of n=5 amino acids in the region from aa 115 to 181 are substituted as compared with the A genotype. In view of the frequency of the amino acid substitutions, it is to be assumed, unexpectedly, that it is a matter of a new mutant or that the mutations are so pronounced that the consequence has more likely to be described as being a new variant, which is designated HDB 05 variant in that which follows.

Analysis of the best agreement of the amino acid sequence of the a determinant with known sequences points to genotype A (Fig. 1), subtype adw (Fig. 2), from which, however, the new variant surprisingly differs in 4 aa positions. The 2 adjacent substitutions

in the region between aa 115 and 120 and between aa 154 and 164 and the aa position # 181 in the direct vicinity of the a determinant, in accordance with Figs. 1, 5 and 6, constitute the most prominent feature.

Since it is known that epitopes on the a determinant are occasioned structurally, that is can be present as what are known as conformational epitopes, it seems likely that the immunogenicity, and also the ability of antibodies to bind to the a determinant, can be influenced by the amino acid substitution in position # 181.

Finally, and entirely unexpectedly, identity was observed between the nucleotide and amino acid sequences of serum sample # 119617 and the corresponding analytical results from another independent serum sample from Austria (internal number: 118457), which likewise originates from a patient who had contracted inflammation of the liver. It can be concluded from this that HDB 05 is a replication-capable and infectious mutant or variant of HBV which may have become disseminated to some degree.

The present invention encompasses an isolated nucleotide sequence which is at least 65% identical with SEQ ID NO: 1 or with a fragment of this sequence depicted in Figs. 3 and 4 which hybridizes specifically with the complement of SEQ ID NO: 1 to 11.

In addition, the present invention encompasses an isolated nucleotide sequence which encodes the present variant according to the invention of the a determinant of the hepatitis B surface antigen (HBsAg) in the amino acid positions between aa 101 and 180 or leads to a peptide product whose aa sequence is in at least 65% agreement with the SEQ ID NO: 12 depicted in Figs. 5 and 6 or fragments thereof in accordance with SEQ ID

NO: 13 to 22.

The present invention furthermore relates to a vector which comprises one or more of said nucleotide sequences as well as to a host cell which harbors this vector and to a method for preparing a corresponding polypeptide from the a determinant, which method comprises incubating the abovementioned host cell over periods and under conditions which are required for expressing the polypeptide.

The invention also relates to antibodies which react with the a determinant described in SEQ ID NO: 11 to 22, with the binding preferably taking place in the amino acid region aa 115 to 120, aa 154 to 164 or aa 154 to 185. The antibodies can be of polyclonal or monoclonal, animal or human origin.

The invention likewise relates to an isolated HBV variant, with the virus possessing an a determinant which corresponds to the aa sequences at least between position 115 and 120 and/or aa 154 to 164 or aa 154 to 181, ideally to all said regions between 115 and 181.

The present invention also relates to an immunogenic mixture for generating polyclonal or monoclonal antibodies, which mixture comprises the described, isolated HBV or one or more of the described polypeptides.

The invention also encompasses a polynucleotide probe which contains an HBV genome sequence which, by substitution of amino acids, leads to a modified a determinant which is identical with the described aa sequence of the novel HBV variant or is in at least 65% correspondence with it.

The invention also relates to kits for detecting

polynucleotides of the HBV variant with the aid of said probe as well as to kits for detecting HBsAg of the variant or individual epitopes thereof and to antibodies which are specific for the variant or epitopes thereof, as well as to the methods for detecting polynucleotides, antigen and antibody, comprising an incubation for forming corresponding complexes and detection of these complexes using suitable methods known to the skilled person.

The embodiments of these kits and detection methods can be designed for the specific and sole detection of nucleotides and antigens of the HBV variant, or of antibodies directed against them, or be supplementary, i.e. permit detection of the variant analyte according to the invention in addition to currently known HBV nucleotides, antigens or antibodies.

In an analogous manner, an immunogenic mixture of polypeptide sequences according to the invention can also be used in combination with known antigens, e.g. for improving the efficacy of the vaccine.

In addition, the present invention is described in the patent claims.

Description of the figures:

Fig. 1 presents an overview of the amino acid sequences of the a determinant of 6 described HBV genotypes in comparison with HDB 05.

Fig. 2 depicts the nucleotide and amino acid sequences of the a determinant, as well as immediately adjacent regions of the HBV genotype A, subtype adw.

Fig. 3 depicts the nucleotide sequence of the a determinant of the HBV surface antigen for subtype

adw of HBV genotype A as compared with the nucleotide sequence of HDB 05.

Fig. 4 summarizes the translation-relevant differences in the nucleotide sequence of HDB 05.

Fig. 5 depicts the nucleotide sequence of HDB 05 in the region of the a determinant, as well as the corresponding amino acid sequence. The a determinant is located between amino acids No. 101 and 180 of the small HBsAg (small, S).

Fig. 6 provides the corresponding polypeptide sequence of the a determinant of HDB 05, which polypeptide sequence is encoded by the nucleotide sequence described in Fig. 5.

The present invention describes a novel variant of the hepatitis B virus (HBV) which possesses an entirely novel a determinant as a result of amino acid substitutions in the following aa positions of the S-HBsAg sequence. The single-letter code is used for describing the amino acids:

aa of HDB 05	aa position	aa of adw/genotype A
R	115	T
Q	120	P
L	154	S
V	164	E

In addition, arginine (R) is present in place of Gln (Q) in position aa 181 of HDB 05:

R	181	Q

These aa substitutions can be attributed to corresponding nucleotide substitutions in the corresponding codons.

The present invention relates to an isolated nucleotide sequence which encodes the a determinant of the virus (Fig. 3 and also SEQ ID NO: 1).

The invention also encompasses nucleotides having at least 65% congruence, preferably at least 75% congruence, and particularly preferably having at least 90% congruence, with the nucleotide sequence of the present invention, or fragments thereof, as well as sequences which are complementary thereto.

Congruence is defined as the degree of alikeness, correspondence or equivalence between two alike strands of two DNA segments (reading either 5'>3'/sense or 3'>5'/antisense). The congruence is expressed as a percentage value by the number of identical bases between two sequences to be compared being divided by the length of the shorter sequence and multiplied by 100, the larger value corresponding to the largest congruence (Smith et al., Adv. Appl. Mathem. 2: 482-489 (1981).

This assessment is also applicable to aa sequences of peptides and proteins (Dayhoff, Atlas of Protein Sequences and Structure, M. O. Dayhoff ed. 5 Suppl. 3: 353-358, Nat. Biom. Res. Found., Washington D.C., USA, Gribskov, Nucl. Acids Res. 14 (6): 6745-66763 (1986).

A person skilled in the art is aware that in addition to the so-called BestFit program from Genetic Computer Group (Madison, WI) there are several analogous evaluation programs for determining the degree of congruence.

Complementarity is to be understood as referring to the degree to which two DNA segments are able to bind. Complementarity is determined by measuring the ability

of a sense strand to hybridize, i.e., to form a double helix, with an antisense strand of another DNA segment. The greater the complementarity between two nucleotide sequences of two DNA strands, the greater the degree will be to which hybrid duplex structures are formed.

The invention also encompasses polypeptides which are encoded by above-described nucleotide sequences, in particular those amino acid sequences which determine the a determinant of the HBsAg, and polypeptides which at least exhibit a similarity of 65%, preferably 75%, and even more preferably 95%, to these sequences.

Similarity between amino acid sequences is defined as the presence of identical amino acids in two polypeptides to be compared. Percentage similarity between two polypeptides is determined using methods familiar to a person skilled in the art, a high percentage denoting high correspondence, alikeness or equivalence of two sequences.

For the description of the present invention, a nucleotide fragment is understood as being a consecutive sequence of at least 9, preferably 9-15, particularly preferably 15-21, and even very particularly preferably 21-60, nucleotides from the nucleotide sequence of the novel HBV variant, with mixtures of these nucleotide fragments also being assumed.

A polypeptide fragment is understood as being a sequence of at least 3, preferably 3-5, particularly preferably 5-7, and even very particularly preferably 7-20, amino acids from the a determinant of the novel HBV variant, with mixtures of such polypeptide fragments also being encompassed by this invention.

The present invention also encompasses an isolated

nucleotide sequence which can be hybridized and leads to nucleotide sequences which correspond to the nucleotide sequences of the HBsAg of the novel HBV variant or parts of the a determinant of the novel HBV variant, are complementary thereto, or are to be traced back to HDB 05 as a subtype or mutation.

It is obvious to the skilled person that, after its isolation using methods in accordance with the prior art, a nucleotide sequence can be introduced into prokaryotic (e.g. E. coli) or eukaryotic host cells (e.g. Chinese hamster ovary cell) or yeast (e.g. S. Cerevisiae) with the aid of a vector or construct (using methods known to the skilled person such as transfection, transformation or electroporation: Molecular Cloning: A Laboratory Manual, 2nd ed., Vol. 1-3, ed Sambrook et al., Cold Spring Harbor Laboratory Press (1989), with it being possible to use transient or permanent cultures.

Consequently, the present invention encompasses isolated nucleotide sequences of the a determinant of the novel HBV variant, polypeptides which are encoded by these nucleotides, vectors which contain nucleotide sequences of the a determinant of the novel HBV variant, and also the host cell into which a vector is introduced.

In addition to using an expression system to prepare polypeptides (recombinantly), it is obvious that analogous polypeptide structures are also prepared synthetically or directly by purification from the virus variant.

It is similarly obvious to the skilled person to use the polypeptides or proteins of the novel HBV variant to generate monoclonal and/or polyclonal antibodies which bind immunologically to binding sites (epitopes)

of the a determinant of the novel HBV variant. The methods for preparing antibodies are known to the skilled person (e.g. Koehler et al., Nature 256-495 (1975), Mimms et al., Vi. 176: 604-619 (1990).

It is furthermore obvious to use the a determinant of the HDB 05 variant according to the invention, in the form of the entire polypeptide sequence or parts thereof, for determining antibodies which are directed against the HBV variant: anti-HBs antibodies.

The skilled person is familiar with a large number of determination methods in which immune complexes are formed, or their formation is inhibited, using polypeptides from the a determinant of the HBV variant and antibodies of animal or human origin.

A special embodiment is the enzyme immunoassay, a possible test principle of which is described below by way of example without, however, restricting the idea of the invention to this principle:

In the very widely used sandwich principle, immobilized epitope-carrying polypeptide or protein sequences are incubated with the sample under investigation on a suitable support (e.g. microparticles or the surface of wells in a microtitration plate). After excess sample has been removed, antibodies which are bound to the epitopes are detected by carrying out a further incubation with epitope-carrying polypeptide or protein sequences which are provided with a probe. The probe employed is frequently an enzyme whose catalytic conversion (after the excess reagent has been removed) of a suitable substrate results in a color reaction which is measured photometrically and whose intensity is proportional to the content of antibody which is present in the sample.

Aside from this special embodiment, methods are also known which are homogeneous in nature (i.e. not require any bound/free separation), which manage entirely without a probe (e.g. agglutination method), which can be evaluated with the naked eye (e.g. radial immunodiffusion) or which make use of other probes (e.g. radioactive isotopes or chemiluminescence) or several probes (e.g. the biotin/streptavidin system). It is likewise possible for the polypeptide structures of the HBV variant to be represented by antiidiotypic antibodies or, by selecting a suitable test principle, for variant-specific monoclonal or polyclonal antibodies to be used for determining anti-HBs antibodies (in a competitive test format). It is likewise known that, by selecting the test principle, it is also possible to differentiate the immunoglobulin classes (e.g. by means of the "indirect" method using a second class-specific antibody (e.g. IgM- or IgG-specific) possessing any probe or with the aid of what is termed the anti-μ principle (IgM-specific). The methods and materials (incl. probe and polypeptide sequences) naturally have to be adapted to the given aim.

All these embodiments correspond to the prior art, such that, in the case of the present invention, "determining antibodies which are specific for the a determinant of the novel HBD 05 variant" is understood as referring to any methods which are suitable for detecting immunoglobulins and/or immunoglobulin classes directed against the novel HBV variant, irrespective of whether the antibody directed against the novel variant is sought on its own or in combination with antibodies directed against known a determinants and/or known mutations in the a region.

Finally, it is similarly obvious to use monoclonal or polyclonal antibodies (or mixtures or fragments thereof or mixtures of fragments) which react with epitopes of

the novel HBV variant to determine the a determinant of the HBV variant according to the invention in the form of the entire polypeptide sequence, or parts thereof, in samples under investigation: HBsAg of the HDB 05 variant.

The skilled person is familiar with a large number of determination methods in which immune complexes are formed, or their formation is inhibited, using one or more monoclonal antibody(ies) or polyclonal antibodies (or mixtures thereof or fragments or mixtures of fragments) which are specific for the a determinant of the HBV variant.

A special embodiment is the enzyme immunoassay, a possible test principle of which is described below by way of example without, however, restricting the idea of the invention to this principle:

In the very widely used sandwich principle, immobilized antibodies, or fragments thereof, are incubated with the sample under investigation on a suitable support (e.g. microparticles or the surface of wells in a microtitration plate). After excess sample has been removed, HBsAg which is bound to the antibodies is detected by carrying out a further incubation with anti-HBs antibodies (monoclonal or polyclonal or fragments or mixtures of these fragments) which are provided with a probe. The probe employed is frequently an enzyme whose catalytic conversion (after the excess reagent has been removed) of a suitable substrate results in a color reaction which is measured photometrically and whose intensity is proportional to the content of antibody which is present in the sample.

Aside from this special embodiment, methods are also known which are homogeneous in nature (i.e. do not required any bound/free separation), which manage

entirely without any probe (e.g. agglutination method), which can be evaluated with the naked eye (e.g. radial immunodiffusion), or which makes use of other probes (e.g. radioactive isotopes or chemiluminescence) or several probes (e.g. the biotin/streptavidin system).

All these embodiments correspond to the prior art, such that, in the case of the present invention, "determining HBsAg of the novel HBV variant" is understood as referring to any methods which are suitable for detecting polypeptide sequences or antigens of the novel HBV variant, irrespective of whether the HBsAg of the novel variant is determined on its own or whether it is determined in combination with HBsAg of known a determinants and/or known mutations in the a region.

It is similarly obvious, for economic reasons, to combine an HBsAg determination with a method for detecting another analyte (e.g. HIV antigen or the simultaneous determination of HBV variant HBsAg and specific antibodies directed against it) in one test assay (which is differentiating or nondifferentiating).

With reference to nucleic acid test (NAT), it is obvious to use nucleotide sequences of the present invention to prepare DNA oligomers of 6-8 nucleotides or more which are suitable for use as hybridization probes for detecting the viral genome of the HBV variant which is described in individuals who are suspected of carrying the virus variant, or, for example in the field of blood donation, for screening stored blood for the presence of the variant genome, either selectively or in combination with detecting nucleotide sequences of known HBV variants and/or HBV mutants.

It is likewise possible to develop corresponding

primers on the basis of the nucleotide sequences of the novel HBV variant which have been found.

In addition, the invention also includes a vaccine which comprises a polypeptide of the present invention and a customary adjuvant (e.g. Freund's adjuvant, phosphate-buffered saline or the like). A vaccine of this nature can be used to stimulate the formation of antibodies in mammals. Similarly, the invention encompasses a particle which comprises a non-variant-specific amino acid sequence which induces particle formation together with an epitope-containing polypeptide which is specific for the HBV variant according to the invention.

Finally, the invention also relates to diagnostic reagents as kits which, based on the above-described methods detection of HBV variant-specific antigen (HBsAg) or antibodies directed against it (anti-HBs), either as single determinations or can be combined with each other or with other known HBV antigens or antibodies which react specifically therewith or else with quite different analytes.

In addition, the present nucleotide sequences can be used for preparing primers and/or gene probes, for which reason kits which comprise primers and/or probes for detecting HBV variant-specific nucleic acid, either on its own or in combination with known HBV nucleotide sequences, in samples under investigation are likewise part of the subject matter of the invention.

Nucleotide sequences of the invention can also be used for preparing antisense oligonucleotides (where appropriate for therapeutic purposes).

Finally, on the basis of the present nucleotide sequences, it is also possible to develop primers which

can be used in the polymerase chain reaction (PCR). PCR is a method for amplifying a desired nucleotide sequence of a nucleic acid or of a nucleic acid mixture. In this method, the primers are in each case extended specifically by a polymerase using the desired nucleic acid as the reading frame. Following dissociation from the original strand, new primers are hybridized and once again extended by the polymerase. By repetition of these cycles, the sought-after target sequence molecules are enriched.

Finally, the invention also comprises cultures of tissue cells which are infected with the HBV variant, as well as the isolated HBV variant itself. An immunogenic preparation which contains the attenuated or inactivated HDB 05 variant of HBV is also part of the subject matter of the invention.

The following examples are intended to describe the present invention, without restricting it to the examples which are described.

Example 1: Using enzyme immunoassay, EIA, to determine HBsAg

The enzyme immunoassay Enzygnost® HBsAg 5.0 from Dade Behring GmbH, Marburg, Germany, was used to determine the HBV surface antigen, i.e. HBsAg, in the blood of the patients from France and Austria.

It is a high-performance test which is approved in Europe and which was performed in accordance with the instructions in the pack information leaflet.

The underlying test principle is a sandwich test in microtiter plate format:

100 µl of the sample to be investigated are brought

into contact, in a one-step method, with 25 µl of conjugate 1 (mouse monoclonal HBsAg-specific antibodies which are covalently labeled with biotin) and immobilized sheep polyclonal HBsAg-specific antibodies. After a 60-minute incubation at 37°C, and after removing excess components by washing the plate wells 4 times, 100 µl of conjugate 2, which consists of streptavidin to which the probe enzyme peroxidase is covalently bonded, are added.
After a 30-minute incubation at 37°C, and after having removed excess components by washing the plate wells 4 times, 75 µl of chromogen buffer/substrate solution are added, with this being followed by a 30-minute incubation at room temperature. The development of the blue tetramethylbenzidine dye is terminated by adding 75 µl of stopping solution (sulfuric acid) and the dye is measured photometrically at 450 nm.

The intensity of the color which develops, as measured by the optical density (O.D.), is directly proportional to the content of HBsAg in the investigated sample, with an O.D. value of less than the threshold value being assessed as HBsAg-negative. The threshold value is defined as the mean value of the O.D. of the negative control (contained in the test kit) which is tested in parallel, to which a constant quantity of 0.05 O.D. is added.

The detection limits of the batch (# 32874) which was used for the investigation were determined, by means of graphic interpolation and using the internationally accepted standard preparations from the Paul Ehrlich Institute, Langen, Germany, to be 0.012 ng of ad subtype/ml and, respectively, 0.015 ng of ay subtype/ml in parallel with the experimental assays from tests of dilutions of the standard preparations in HBsAg-negative serum.
Analysis of the samples # 119617 and 118234 from which

the DNA was also isolated, gave results, for both samples of between 0.02 and 0.05 O.D. in 2 independent experiments on two different days, which results are to be interpreted, in accordance with the criteria of the test, as being HBsAg-negative. On the other hand, the positive control (contained in the test kit) which was concomitantly assayed was as positive (validation criteria fulfilled) as the abovementioned ad and ay standard preparations.

Example 2: Isolating the HDB 05 DNA from sample # 118234

The QIA amp® DNA blood mini kit from Qiagen, Hilden, Germany, was used to isolate the DNA from in each case a 200 µl aliquot of the French and Austrian samples. In doing this, all the procedural steps were followed as described in the pack information leaflet and the elution was performed in a volume of 50 µl in each case.

Example 3: Polymerase chain reaction, PCR

3.1 **HBV primers**

The four HBV primers listed below were used:

Primer 1 having the 5'>3' sequence:
GGGTCACCATATTCTTGGGAAC
Primer 2 having the 5'>3' sequence:
TATACCCAAAGACAAAAGAAAATTGG
Primer 3 having the 5'>3' sequence:
GACTCGTGGTGGACTTCTCTC
Primer 4 having the 5'>3' sequence:
TACAGACTTGGCCCCCAATACC

3.2 **PCR amplification**

The Perkin Elmer Ampli Taq ® DNA polymerase kit as well as the Thermocycler Gene Amp ® PCR system

9700 from Perkin Elmer Applied Biosystems, USA, were used to carry out a nested PCR amplification of the surface antigen.
The nucleotides were obtained from Amersham Biosciences, UK.

For the first amplification cycle, 5 µl of the isolated DNA were amplified using the abovementioned primers 1 and 2 and the following conditions:

PCR 1 rxn

Primer 1 (10 µM)	1 µl	
Primer 2 (10 µM)	1 µl	
10-fold conc. buffer		
(incl. 15 µM Mg2Cl)	5 µl	
dNTP mixture (10 µM)	1 µl	
dist. Water	36.75 µl	
Ampli Taq (5 U/µl)	0.25 µl	
per tube	45 µl	total volume
plus	5 µl	of isolated DNA
	50 µl	reaction volume

The 50 µl assay was amplified using the above-described thermocycler under the following conditions:

94°C, 1 min./94°C, 28 sec. - 55°C, 28 sec. - 72°C, 38 sec. (35 cycles)/72°C, 5 min./8°C soak.

In the second round of amplification, 5 µl of the first PCR product were further amplified using the HBV primers 3 and 4 and the following conditions:

PCR 2 rxn

Primer 3 (10 μM)	1 μl
Primer 4 (10 μM)	1 μl
10-fold conc. buffer	5 μl
dNTP mixture (10 μM)	1 μl
dist. Water	36.75 μl
Ampli Taq (5 U/μl)	0.25 μl
per tube	45 μl total volume
plus	5 μl of PCR product v.rxn
	50 μl reaction volume

This PCR 2 assay was amplified using the above-described thermocycler and employing the following conditions:

94°C, 1 min./94°C, 28 sec. - 55°C, 28 sec. - 72°C, 38 sec. (35 cycles)/72°C, 5 min./8°C soak.

In conclusion, the PCR 2 product was fractionated electrophoretically (1.5% agarose) while including suitable molecular weight markers. The band containing approx. 520 base pairs was excised and isolated using the QIA quick gel extraction kit from Qiagen, Hilden, Germany.

Example 4: Sequencing HDB 05

The purified PCR product was sequenced by Medigenomix, Martinsried, Germany, with the aid of the ABI 3700 Kapillar system in combination with the ABI BigDye Terminator Chemistry Version 1.1. and the ABI Sequencing Analysis Software Version 3.6. and using the primers 3 and 4 described in Ex. 3.

Sequencing results

It was shown that the HBsAgs of the two analyzed

samples agreed with each other and that, within the sequenced region, the nucleotide and amino acid sequences exhibited the best agreement with genotype A, subtype adw. In agreement with each other, the analyzed samples from France and Austria exhibited a total of 4 amino acid substitutions in the region of the a determinant as compared with genotype A, subtype adw (see also Figs. 2 and 5):

HDB 05		**A, adw**
1.)	Arg (R)	substituted for 115 Thr (T)
2.)	Gln (Q)	substituted for 120 Pro (P)
3.)	Leu (L)	substituted for 154 Ser (S)
4.)	Val (V)	substituted for 164 Glu (E)

In addition, there is an amino acid substitution at position # 181:

5.)	Arg (R)	substituted for 181 Gln (Q).

These results were reproduced, with the same sequencing results, in several independent analyses of the two investigated blood samples from France and Austria, with the sequencing results furthermore exhibiting complete agreement in the case of the two independent samples.

Patent Claims:

1. An isolated oligonucleotide or polynucleotide having one of the sequences selected from the group consisting of SEQ ID NO: 1 to SEQ ID NO: 11:

SEQ ID No: 1

```
127  GGGGGATCAC CCGTGTGTCT TGGCCAAAAT TCGCAGTCCC CAACCTCCAA
     TCACTCACCA ACCTCCTGTC CTCCAATTTG TCCTGGTTAT CGCTGGATGT
     GTCTGCGGCG TTTTATCATA TTCCTCTTCA TCCTGCTGCT ATGCCTCATC
     TTCTTATTGG TTCTTCTGGA TTATCAAGGT ATGTTGCCCG TTTGTCCTCT
     AATTCCAGGA TCAACAAGAA CCAGTACGGG ACAATGCAAA ACCTGCACGA
     CTCCTGCTCA AGGCAACTCT ATGTTTCCCT CATGTTGCTG TACAAAACCT
     ACGGATGGAA ATTGCACCTG TATTCCCATC CCATTGTCCT GGGCTTTCGC
     AAAATACCTA TGGGTGTGGG CCTCAGTCCG TTTCTCTTGG CTCAGTTTAC
     TAGTGCCATT TGTTCGGTGG TTCGTAGGGC TTTCCCCCAC TGTTTGGCTT
     TCAGCTATAT GG   588
```

SEQ ID No: 2

```
331  CCAGGATCAA CAAGAACCAG TACGGGACAA TGCAAAACCT GCACGACTCCT
     GCTCAAGGCA ACTCTATGTT TCCCTCATGT TGCTGTACAA AACCTACGGA
     TGGAAATTGC ACCTGTATTC CCATCCCATT GTCCTGGGCT TTCGCAAAAT
     ACCTATGGGT GTGGGCCTCA GTCCGTTTCT CTTGGCTCAG TTTACTAGTG
     CCATTTGTTC GGTGGTTCGT AGGG   555
```

SEQ ID No: 3

```
331  CCAGGATCAA CAAGAACCAG TACGGGACAA TGCAAAACCT GCACGACTCC
     TGCTCAAGGC AACTCTATGT TTCCCTCATG TTGCTGTACA AAACCTACGG
     ATGGAAATTGC ACCTGTATT CCCATCCCAT TGTCCTGGGC TTTCGCAAAA
     TACCTATGGG TGTGGGCCTC AGTCCGTTTC  510
```

SEQ ID No: 4

```
331  CCAGGATCAA CAAGAACCAG TACGGGACAA TGCAAAACCT GCACGACTCC
     TGCTCAAGGC AACTCTATGT TTCCCTCATG TTGCTGTACA AAACCTACGG
     ATGGAAATTG CACCTGTATT CCCATCCCAT TGTCCTGGGC TTTCGCAAAA
     TACCTATGGG
     TGTGG  495
```

SEQ ID No: 5

```
331  CCAGGATCAA CAAGAACCAG TACGGGACAA TGCAAAACCT GCACGACTCC
     TGCTCAAGGC   390
```

SEQ ID No: 6

```
331  CCAGGATCAA CAAGAACCAG TACGGGACAA   360
```

SEQ ID No: 7

343 AGAACCAGTA CGGGACAATG CAAAACCTGC ACGACTCCTG CTCAAGGCAA
CTCTATGTTT CCCTCATGTT GCTGTACAAA ACCTACGGAT GGAAATTGCA
CCTGTATTCC CATCCCATTG TCCTGGGCTT TCGCAAAATA CCTATGGGTG
TGG 495

SEQ ID No: 8

343 AGAACCAGTA CGGGACAA 360

SEQ ID No: 9

460 TTGTCCTGGG CTTTCGCAAA ATACCTATGG GTGTGGGCCT CAGTCCGTTT
CTCTTGGCTC AGTTTACTAG TGCCATTTGT TCGGTGGTTC GTAGGG 555

SEQ ID No: 10

460 TTGTCCTGGG CTTTCGCAAA ATACCTATGG GTGTGGGCCT CAGTCCGTTT
C 510

SEQ ID No: 11

462 TTGTCCTGGG CTTTCGCAAA ATACCTATGG GTGTGG 495

2. The oligonucleotide or polynucleotide as claimed in claim 1, which is in each case at least 65% or 66% or 67% or 68% or 69% or 70% or 71% or 72% or 73% or 74% or 75% or 76% or 77% or 78% or 79% or 80% or 81% or 82% or 83% or 84% or 85% or 86% or 87% or 88% or 89% or 90% or 91% or 92% or 93% or 94% or 95% or 99% or 97% or 98% or 99% identical with one of the sequences selected from the group consisting of SEQ ID NO: 1 to SEQ ID NO: 11.

3. The oligonucleotide or polynucleotide as claimed in claim 1 or 2, which hybridizes, under stringent conditions, with an oligonucleotide or polynucleotide which has a sequence which is complementary to one of the sequences selected from the group consisting of SEQ ID NO: 1 to SEQ ID NO: 11.

4. An isolated oligonucleotide or polynucleotide which encodes HBs antigen of the hepatitis B virus and contains an oligonucleotide or polynucleotide

as claimed in one of claims 1 to 3.

5. A fragment of an oligonucleotide or polynucleotide which encodes HBs antigen of the hepatitis B virus, wherein the fragment contains an oligopeptide or polypeptide as claimed in one of claims 1 to 3.

6. An isolated oligonucleotide or polynucleotide which encodes the a determinant of the HBs antigen of the hepatitis B virus and contains an oligonucleotide or polynucleotide as claimed in one of claims 1 to 3.

7. A primer which is specific for an oligonucleotide or polynucleotide as claimed in one of claims 1 to 6.

8. A vector which contains at least one oligonucleotide or polynucleotide as claimed in one of claims 1 to 5.

9. A host cell which harbors a vector as claimed in claim 8.

10. An oligopeptide or polypeptide which is encoded by an oligonucleotide or polynucleotide as claimed in one of claims 1 to 5.

11. An isolated oligopeptide or polypeptide which has an amino acid sequence which is selected from the group consisting of SEQ ID NO: 12 to SEQ ID NO 22:

SEQ ID NO.: 12
43 G G S P V C L G Q N S Q S P T S N H
S P T S C P P I C P G Y R W M C L R R F
I I F L F I L L L C L I F L L V L L D Y
Q G M L P V C P L I P G S T R T S T G Q
C K T C T T P A Q G N S M F P S C C C T
K P T D G N C T C I P I P L S W A F A K
Y L W V W A S V R F S W L S L L V P F V
R W F V G L S P T V W L S A I W 196

SEQ ID NO.: 13
111 P G S T R T S T G Q C K T C T T P A
Q G N S M F P S C C C T K P T D G N C T
C I P I P L S W A F A K Y L W V W A S V
R F S W L S L L V P F V R W F V G 185

SEQ ID NO.: 14
111 P G S T R T S T G Q C K T C T T P A
Q G N S M F P S C C C T K P T D G N C T
C I P I P L S W A F A K Y L W V W A S V
R F 170

SEQ ID NO.: 15
111 P G S T R T S T G Q C K T C T T P A
Q G N S M F P S C C C T K P T D G N C T
C I P I P L S W A F A K Y L W V W 165

SEQ ID NO.: 16
111 P G S T R T S T G Q C K T C T T P A
Q G 130

SEQ ID NO.: 17
111 P G S T R T S T G Q 120

SEQ ID NO.: 18
115 R T S T G Q C K T C T T P A Q G N S
M F P S C C C T K P T D G N C T C I P I
P L S W A F A K Y L W V W 165

SEQ ID NO.: 19
115: R T S T G Q 120

SEQ ID NO.: 20
154 P I P L S W A F A K Y L W V W A S V R
F S W L S L L V P F V R W F V G L 185

SEQ ID NO.: 21
154 P I P L S W A F A K Y L W V W A S V R F 170

SEQ ID NO.: 22
154: P I P L S W A F A K Y L W V W 165

12. An oligopeptide or polypeptide as claimed in claim 10 or 11 which is in each case at least 65% or 66% or 67% or 68% or 69% or 70% or 71% or 72% or 73% or 74% or 75% or 76% or 77% or 78% or 79% or 80% or 81% or 82% or 83% or 84% or 85% or 86% or 87% or 88% or 89% or 90% or 91% or 92% or 93% or 94% or 95% or 99% or 97% or 98% or 99% identical with one of the sequences selected from the group consisting of SEQ ID NO: 12 to SEQ ID NO: 22.

13. An isolated polypeptide corresponding to the sequence of the HBs antigen of the hepatitis B virus, wherein it contains an oligopeptide or polypeptide as claimed in one of claims 10 to 12.

14. A fragment of a polypeptide which corresponds to the sequence of the HBs antigen of the hepatitis B virus, wherein the fragment contains an oligopeptide or polypeptide as claimed in one of claims 10 to 12.

15. An isolated polypeptide which encodes the a determinant of the HBs antigen of the hepatitis B virus, wherein it contains an oligopeptide or polypeptide as claimed in one of claims 10 to 12.

16. A monoclonal or polyclonal antibody which binds to HBs antigen containing an oligopeptide or polypeptide as claimed in one of claims 10 to 15 but which does not bind, or at least binds significantly more weakly, to HBs antigen

belonging to a hepatitis B wild-type virus.

17. An antiidiotypic antibody which represents an amino acid sequence as claimed in one of claims 10 to 15.

18. A test kit for detecting or determining, by means of a hybridization reaction, a nucleic acid which is specific for a variant or mutant of the hepatitis B virus using at least one oligonucleotide or polynucleotide as claimed in one of claims 1 to 7.

19. A test kit for immunochemically detecting or immunochemically determining an antigen which is specific for a variant or mutant of the hepatitis B virus using at least one monoclonal or polyclonal antibody as claimed in claim 16.

20. A test kit for immunochemically detecting or immunochemically determining an antibody directed against a variant or mutant of the hepatitis B virus using at least one oligopeptide or polypeptide as claimed in one of claims 10 to 15.

21. An immunogenic peptide or mixture of immunogenic peptides containing one or more oligopeptides or polypeptides as claimed in one or more of claims 3 and 4 on its/their own or in combination with known HBV immunogens.

Abstract:

Novel surface protein (HBsAg) variant of the hepatitis B virus

---

The invention relates to sequences of a novel variant of the hepatitis B surface antigen (HBsAg) and to methods for detecting nucleic acids, antigens and antibodies thereto in patient samples.

Fig. 1: Amino acid sequence of the HBsAg a determinant of the different HBV genotypes as compared with the novel mutant HDB 05
A representative genome was used as the basis for each genotype and the aa sequence was deduced from the nucleotide sequence
A: X70 185; B: DO0331; C: XO1587; D: X72702, E: X75664; F: X75663; (Stuyver et al.; J. Gen. Virol. 81: 67-74 (2000); Norder et al.: J. Gen. Virol. 73: 3141-3145 (1992))

aa #	101	111	121	131	141	151	161 170
Genotype							
A	QGMLPVCPLI	PGSTTTSTGP	CKTCTTPAQG	NSMFPSCCCT	KPTDGNCTCI	PIPSSWAFAK	YLWEWASVRF
B	----------	---S------	----------	T---------	----------	----------	----------
C	---------L	--TS------	-----I----	T---------	--S-------	---------R	F--G------
D	----------	---S------	-R--------	T--Y------	--S-------	--------G-	F------A--
E	----------	---S------	-R--M-L---	T--------S	--S-------	--------G-	F------A--
F	----------	----------	------L---	T--------S	--S-------	-------LG-	-------A--
HDB 05	----------	----R----Q	----------	----------	----------	---L------	---V------
aa #		115 120				154	164

The amino acid substitutions which differ from the wild-type adw HBV are printed in bold type

Fig. 2 Nucleotide sequence of the S gene of the known HBV adw wild type encoding the HBV surface protein (surface antigen, HBsAg), and resulting amino acid sequence in the 3-letter and, especially, 1-letter codes (Coleman et al.; WO 02/079217 A1)

Continuous numbering of nucleotides (nt) encoding the surface antigen (excl. pre S1 and pre S2 regions)

Continuous numbering of amino acids (aa)

```
                                                                                       (nt)
                                                                                  (aa)
1     ATG GAG AAC ATC ACA TCA GGA TTC CTA GGA CCC CTG CTC GTG TTA CAG GCG GGG TTT TTC        60
  1   Met Glu Asn Ile Thr Ser Gly Phe Leu Gly Pro Leu Leu Val Leu Gln Ala Gly Phe Phe   20
       M   E   N   I   T   S   G   F   L   G   P   L   L   V   L   Q   A   G   F   F
61    TTG TTG ACA AGA ATC CTC ACA ATA CCG CAG AGT CTA GAC TCG TGG TGG ACT TCT CTC AAT        120
 21   Leu Leu Thr Arg Ile Leu Thr Ile Pro Gln Ser Leu Asp Ser Trp Trp Thr Ser Leu Asn   40
       L   L   T   R   I   L   T   I   P   Q   S   L   D   S   W   W   T   S   L   N
121   TTT CTA GGG GGA TCA CCC GTG TGT CTT GGC CAA AAT TCG CAG TCC CCA ACC TCC AAT CAC        180
 41   Phe Leu Gly Gly Ser Pro Val Cys Leu Gly Gln Asn Ser Gln Ser Pro Thr Ser Asn His   60
       F   L   G   G   S   P   V   C   L   G   Q   N   S   Q   S   P   T   S   N   H
181   TCA CCA ACC TCC TGT CCT CCA ATT TGT CCT GGT TAT CGC TGG ATG TGT CTG CGG CGT TTT        240
 61   Ser Pro Thr Ser Cys Pro Pro Ile Cys Pro Gly Tyr Arg Trp Met Cys Leu Arg Arg Phe   80
       S   P   T   S   C   P   P   I   C   P   G   Y   R   W   M   C   L   R   R   F
241   ATC ATA TTC CTC TTC ATC CTG CTG CTA TGC CTC ATC TTC TTA TTG GTT CTT CTG GAT TAT        300
 81   Ile Ile Phe Leu Phe Ile Leu Leu Leu Cys Leu Ile Phe Leu Leu Val Leu Leu Asp Tyr   100
       I   I   F   L   F   I   L   L   L   C   L   I   F   L   L   V   L   L   D   Y
301   CAA GGT ATG TTG CCC GTT TGT CCT CTA ATT CCA GGA TCA ACA ACA ACC AGC ACG GGA CCC        360
101   Gln Gly Met Leu Pro Val Cys Pro Leu Ile Pro Gly Ser Thr Thr Thr Ser Thr Gly Pro   120
       Q   G   M   L   P   V   C   P   L   I   P   G   S   T   T   T   S   T   G   P
361   TGC AAA ACC TGC ACG ACT CCT GCT CAA GGA AAC TCT ATG TTT CCC TCC TGT TGC TGT ACA        420
121   Cys Lys Thr Cys Thr Thr Pro Ala Gln Gly Asn Ser Met Phe Pro Ser Cys Cys Cys Thr   140
       C   K   T   C   T   T   P   A   Q   G   N   S   M   F   P   S   C   C   C   T
421   AAA CCT ACG GAT GGA AAC TGC ACC TGT ATT CCC ATC CCA TCA TCC TGG GCT TTC GCA AAA        480
141   Lys Pro Thr Asp Gly Asn Cys Thr Cys Ile Pro Ile Pro Ser Ser Trp Ala Phe Ala Lys   160
       K   P   T   D   G   N   C   T   C   I   P   I   P   S   S   W   A   F   A   K
481   TAC CTA TGG GAG TGG GCC TCA GTC CGT TTC TCT TGG CTC AGT TTA CTA GTG CCA TTT GTT        540
161   Tyr Leu Trp Glu Trp Ala Ser Val Arg Phe Ser Trp Leu Ser Leu Leu Val Pro Phe Val   180
       Y   L   W   E   W   A   S   V   R   F   S   W   L   S   L   L   V   P   F   V
541   CAA TGG TTC GTA GGG CTT TCC CCC ACT GTT TGG CTT TCA GCT ATA TGG ATG ATG TGG TAT        600
181   Gln Trp Phe Val Gly Leu Ser Pro Thr Val Trp Leu Ser Ala Ile Trp Met Met Trp Tyr   200
       Q   W   F   V   G   L   S   P   T   V   W   L   S   A   I   W   M   M   W   Y
601   TGG GGG CCA AGA CTG TAC TCC ATC GTT AGT CCC TTT ATC CCG CTG TTA CCA ATT TTC TTT        660
201   Trp Gly Pro Arg Leu Tyr Ser Ile Val Ser Pro Phe Ile Pro Leu Leu Pro Ile Phe Phe   220
       W   G   P   R   L   Y   S   I   V   S   P   F   I   P   L   L   P   I   F   F
661   TGT CTT TGG GTA TAC ATT  678
221   Cys Leu Trp Val Tyr Ile  226/389
       C   L   W   V   Y   I
```

Fig. 3 **Nucleotide sequence of the HBV surface antigen-encoding S gene** of the HBV adw wild type (upper row from nt 1 to nt 678) as compared with the nucleotide sequence, which is sequenced from nt 127 to nt 588, of the novel variant HDB 05 (lower row, in which nucleotide differences are printed in bold type and bracketed when the mutations do not lead to any amino acid substitution)

1	ATG GAG AAC ATC ACA TCA GGA TTC CTA GGA CCC CTG CTC GTG TTA CAG GCG GGG TTT TTC	60
61	TTG TTG ACA AGA ATC CTC ACA ATA CCG CAG AGT CTA GAC TCG TGG TGG ACT TCT CTC AAT	120
121	TTT CTA GGG GGA TCA CCC GTG TGT CTT GGC CAA AAT TCG CAG TCC CCA ACC TCC AAT CAC 127: GGG GGA TCA CCC GTG TGT CTT GGC CAA AAT TCG CAG TCC CCA ACC TCC AAT CAC	180
182	TCA CCA ACC TCC TGT CCT CCA ATT TGT CCT GGT TAT CGC TGG ATG TGT CTG CGG CGT TTT TCA CCA ACC TCC TGT CCT CCA ATT TGT CCT GGT TAT CGC TGG ATG TGT CTG CGG CGT TTT	240
242	ATC ATA TTC CTC TTC ATC CTG CTG CTA TGC CTC ATC TTC TTA TTG GTT CTT CTG GAT TAT ATC ATA TTC CTC TTC ATC CTG CTG CTA TGC CTC ATC TTC TTA TTG GTT CTT CTG GAT TAT	300
302	CAA GGT ATG TTG CCC GTT TGT CCT CTA ATT CCA GGA TCA ACA ACA ACC AGC ACG GGA CCC CAA GGT ATG TTG CCC GTT TGT CCT CTA ATT CCA GGA TCA ACA **AGA** ACC (AGT) ACG GGA **CAA**	360
361	TGC AAA ACC TGC ACG ACT CCT GCT CAA GGA AAC TCT ATG TTT CCC TCC TGT TGC TGT ACA TGC AAA ACC TGC ACG ACT CCT GCT CAA (GGC) AAC TCT ATG TTT CCC (TCA) TGT TGC TGT ACA	420
421	AAA CCT ACG GAT GGA AAC TGC ACC TGT ATT CCC ATC CCA TCA TCC TGG GCT TTC GCA AAA AAA CCT ACG GAT GGA(AAT) TGC ACC TGT ATT CCC ATC CCA **TTG** TCC TGG GCT TTC GCA AAA	480
481	TAC CTA TGG GAG TGG GCC TCA GTC CGT TTC TCT TGG CTC AGT TTA CTA GTG CCA TTT GTT TAC CTA TGG **GTG** TGG GCC TCA GTC CGT TTC TCT TGG CTC AGT TTA CTA GTG CCA TTT GTT	540
541	CAA TGG TTC GTA GGG CTT TCC CCC ACT GTT TGG CTT TCA GCT ATA TGG ATG ATG TGG TAT **CGG** TGG TTC GTA GGG CTT TCC CCC ACT GTT TGG CTT TCA GCT ATA TGG 588	600
601	TGG GGG CCA AGA CTG TAC TCC ATC GTT AGT CCC TTT ATC CCG CTG TTA CCA ATT TTC TTT	660
661	TGT CTT TGG GTA TAC ATT 678	

Fig. 4 **Nucleotide sequence of the S gene of the novel HBV variant HDB 05: (nt 127 to nt 588) of the HBV surface antigen-encoding genome. Only the nucleotide differences which lead to a change in the amino acid sequence are printed in bold.**

127	GGG GGA TCA CCC GTG TGT CTT GGC CAA AAT TCG CAG TCC CCA ACC TCC AAT CAC	180
181	TCA CCA ACC TCC TGT CCT CCA ATT TGT CCT GGT TAT CGC TGG ATG TGT CTG CGG CGT TTT	240
241	ATC ATA TTC CTC TTC ATC CTG CTG CTA TGC CTC ATC TTC TTA TTG GTT CTT CTG GAT TAT	300
301	CAA GGT ATG TTG CCC GTT TGT CCT CTA ATT CCA GGA TCA ACA **AGA** ACC AGT ACG GGA **CAA**	360
361	TGC AAA ACC TGC ACG ACT CCT GCT CAA GGC AAC TCT ATG TTT CCC TCA TGT TGC TGT ACA	420
421	AAA CCT ACG GAT GGA AAT TGC ACC TGT ATT CCC ATC CCA **TTG** TCC TGG GCT TTC GCA AAA	480
481	TAC CTA TGG **GTG** TGG GCC TCA GTC CGT TTC TCT TGG CTC AGT TTA CTA GTG CCA TTT GTT	540
541	**CGG** TGG TTC GTA GGG CTT TCC CCC ACT GTT TGG CTT TCA GCT ATA TGG **588**	

Fig. 5 **S gene nucleotide sequence (nt 127 to 588) and corresponding amino acid sequence (aa 43 to 196) of the novel HBV variant HDB 05 (amino acids which are substituted as compared with the HBV adw wild type are printed in bold and underlined)**

```
127        GGG GGA TCA CCC GTG TGT CTT GGC CAA AAT TCG CAG TCC CCA ACC TCC AAT CAC    180
     aa 43  G   G   S   P   V   C   L   G   Q   N   S   Q   S   P   T   S   N   H     60

181        TCA CCA ACC TCC TGT CCT CCA ATT TGT CCT GGT TAT CGC TGG ATG TGT CTG CGG CGT TTT    240
     61     S   P   T   S   C   P   P   I   C   P   G   Y   R   W   M   C   L   R   R   F     80

243        ATC ATA TTC CTC TTC ATC CTG CTG CTA TGC CTC ATC TTC TTA TTG GTT CTT CTG GAT TAT    300
     81     I   I   F   L   F   I   L   L   L   C   L   I   F   L   L   V   L   L   D   Y     100

303        CAA GGT ATG TTG CCC GTT TGT CCT CTA ATT CCA GGA TCA ACA AGA ACC AGT ACG GGA CAA    360
     101    Q   G   M   L   P   V   C   P   L   I   P   G   S   T   R   T   S   T   G   Q     120

361        TGC AAA ACC TGC ACG ACT CCT GCT CAA GGC AAC TCT ATG TTT CCC TCA TGT TGC TGT ACA    420
     121    C   K   T   C   T   T   P   A   Q   G   N   S   M   F   P   S   C   C   C   T     140

421        AAA CCT ACG GAT GGA AAT TGC ACC TGT ATT CCC ATC CCA TTG TCC TGG GCT TTC GCA AAA    480
     141    K   P   T   D   G   N   C   T   C   I   P   I   P   L   S   W   A   F   A   K     160

481        TAC CTA TGG GTG TGG GCC TCA GTC CGT TTC TCT TGG CTC AGT TTA CTA GTG CCA TTT GTT    540
     161    Y   L   W   V   W   A   S   V   R   F   S   W   L   S   L   L   V   P   F   V     180

541        CGG TGG TTC GTA GGG CTT TCC CCC ACT GTT TGG CTT TCA GCT ATA TGG   588
     181    R   W   F   V   G   L   S   P   T   V   W   L   S   A   I   W    aa 196
```

The following aa are substituted (x) in the HDB 05 variant as compared with the HBV adw wild type: T 115 (R), P 120 (Q), S 154 (L), E 164 (V) (all in the region of the a determinant) and Q 181 (R) (not in the region of the a determinant).

Fig. 6 Comparison of the amino acid sequences of the a determinant (aa 100 to aa 180) of the novel variant HDB 05 (lower row) and of the HBV adw wild type (upper row)

	aa-Sequence adw wild type	Y	100																		
	aa-Sequence HDB 05 variant	Y																			
101	Q	G	M	L	P	V	C	P	L	I	P	G	S	T	T	T	S	T	G	P	120
	Q	G	M	L	P	V	C	P	L	I	P	G	S	T	R	T	S	T	G	Q	
121	C	K	T	C	T	T	P	A	Q	G	N	S	M	F	P	S	C	C	C	T	140
	C	K	T	C	T	T	P	A	Q	G	N	S	M	F	P	S	C	C	C	T	
141	K	P	T	D	G	N	C	T	C	I	P	I	P	S	S	W	A	F	A	K	160
	K	P	T	D	G	N	C	T	C	I	P	I	P	L	S	W	A	F	A	K	
161	Y	L	W	E	W	A	S	V	R	F	S	W	L	S	L	L	V	P	F	V	180
	Y	L	W	V	W	A	S	V	R	F	S	W	L	S	L	L	V	P	F	V	
181	Q	W	F	V	G	L	S	P	T	V	190										
	R	W	F	V	G	L	S	P	T	V											

The following aa are substituted (x) in the HDB 05 variant as compared with the HBV adw wild type:
T 115 (R), P 120 (Q), S 154 (L), E 164 (V) - (all in the region of the a determinant) and Q 181 (R) (not in the region of the a determinant)